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ENGINEERING OF MICROBIAL PROTEASES: IMPROVING STABILITY AND CATALYTIC PERFORMANCES

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ABSTRACT

Proteolytic enzymes are becoming more ubiquitous as bacteria and fungi. Some proteases from microbial sources are industrially important enzymes but often have to be improved for their catalytic efficiency and stabilities in solvents and temperature at industrial scales in order to suit their applications. Research on protein engineering of microbial proteases to improve their stability and catalytic performances have been extensively conducted by various researchers across the globe using different molecular approaches vis a vis site-directed mutagenesis (SDM) and directed evolutions (DE). SDM has been extremely useful in substitution of important amino acids of microbial proteases; though its major obstacle is that it is imperative to know the three dimensional (3D) structure of the protease in question. Directed evolutions (DE) subsequently emerged as an alternative to SDM, since the knowledge of the enzymes 3D structure is less significant, though its major drawback has been the creation of large mutant libraries and high through put screening of mutant with desired properties. To overcome the drawback of DE, a flow cytometry based screening system have been recently developed which may likely pave way for efficient and fastest way of screening of mutants with improved desired properties.. Sometimes these two approaches can be applied concurrently to obtain enzymes with novel properties. This review aimed at gathering the disperse literature on the approaches where bacteria and fungi have been chosen as sources of microbial proteases. A recent flow cytometry based screening system for DE of proteases has also been reported.

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[I] INTRODUCTION

Many industrial applications of proteases require enzymes with properties that are non physiological. Protein engineering pave ways for the introduction of predesigned changes into the gene for the synthesis of a protein with an altered function that is desired for designated industrial application. Advances seen in recombinant DNA technology and the ability to selectively exchange amino acids by site-directed mutagenesis (SDM) have been responsible for the alarming progress of protein engineering. Identification of the gene and in-depth knowledge of the three-dimensional structure of the protein in question are the two main prerequisites for rational design aspect of protein engineering. The X-ray crystallographic structures of several proteases have been determined [1, 2, 3]. However, because not all proteins and / or enzymes that the scientist have the knowledge of their gene and three-dimensional structures, directed evolution (DE) emerged as a new promising aspect of protein engineering, where the fundamental knowledge of the gene and the 3D structure is less important. Proteases from bacteria and fungi have been engineered to improve their stability and/or catalytic properties to suit their particular applications.

[II] SITE DIRECTED MUTAGENESIS

This type of mutagenesis is also known as oligonucleotide directed mutagenesis or site-specific mutagenesis, is a molecular biology technique in which a mutation is created at a define site in a DNA molecule. The peculiar feature of this mutagenesis requires that the wild type gene be known. Bacteria and fungi are chosen for this review.

2.1. Bacteria

Subtilisin has been selected as a model system for protein engineering since a lot of fundamental information about this commercially important enzyme is available. Its pH dependence [4] catalytic activity [5, 6]stability to heat or denaturing agents [7, 8], and substrate specificity [9, 10, 11, 12, 13] has been altered through SDM. A slightly reduced rate

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of thermal inactivation was observed for a subtilisin BPN9 variant containing two cysteine residues (Cys22, Cys87) [14, 15]. Oxidation of Met222 adjacent to the Ser221 in the active site of subtilisin reduces the catalytic activity of subtilisin.

The effect of substitution of Met222 with different amino acids revealed that small side chains yield the highest activity. The mutant enzymes Ser222, Ala222, and Leu222 were active and stable to peroxide for 1 h. Probing of the specificity of the S1binding site of Met222 Cys/Ser mutants of subtilisin from *Bacillus lentus* with boronic acid inhibitors revealed similar binding trends for the mutant and the parent [16]. The disulfide bonds introduced into subtilisin away from its catalytic center were shown to possess increased autolytic stability [17]. Higher thermostability of subtilisin E as a result of introduction of a disulfide bond engineered on the basis of structural comparison with a thermophilic serine protease has been reported [18]. Strausberg et al. has created the environment for stabilization of subtilisin by deleting the calcium-binding loop from the protein [19]. Analysis of the structure and stability of the prototype with the loop deleted followed by SDM resulted in a mutant with native proteolytic activity and 1,000-fold-greater stability under strongly chelating conditions. SDM-mediated substitution of Asn241 buried in the neutral protease of *B. stearothermophilus* by leucine resulted in an increase in thermostability of 0.7 ± 0.1°C [20]. The thermostability of the neutral protease from *Bacillus subtilis* was increased by 0.3 and 1.0°C by replacing Lys with Ser at positions 249 and 290, respectively, whereas the Asp249 and Asp290 mutants exhibited an increased stabilization by 0.6 and 1.2°C, respectively [21].

A protein engineering study was undertaken by Bruinenberg *et al*,[22] to determine the functions of one of the largest loop insertions (residues 205 to 219), predicted to be spatially close to the substrate-binding region of the SK11 protease from *L. delbrueckii* and susceptible to autoproteolysis [22]. Deletion or modification of this loop was shown to affect the activity and autoprocessing of the protease. Graham *et al,* [23] showed that random mutagenesis of the substrate-binding site of a-lytic protease, a serine protease secreted by the soil bacterium *Lysobacter enzymogenes*, generated enzymes with increased activities and altered primary specifities Substitution of His120 by Ala in the LasA protease of *Pseudomonas aeruginosa* yielded an enzyme devoid of staphylolytic activity. Thus, His120 was shown to be essential for LasA activity [24].

2.2. Fungi

Fungal aspartic proteases are able to cleave substrate with "Lys" in the P1 position. Sequencing and structural comparison suggest that two aspartic acid residues (Asp30 and Asp77) may be responsible for conferring this unique specificity [25].Estell *etal*,engineered the substrate specificity of rhizopus pepsin from *Rhizopus niveus* and demonstrated the role of Asp77 in the hydrolysis of the substrates with lysine in the P-1 position [25]. The primary structure of aspergillopepsin I from *Aspergillus saitoi* ATCC 14332 (now designated A. phoenicis) was deduced from the nucleotide sequence of the gene [26]. To identify the residue responsible for determining the specificity of aspergillopepsin I toward the basic substrates in the substrate-binding pocket, Asp76 was replaced with a Ser residue by SDM. The striking feature of this mutation was that only the trypsinogen activating activity of the enzyme was destroyed, suggesting the importance of the Asp76 residue in binding to basic substrates. To elucidate whether the processing of the pro-region occurs by autoproteolysis or by involving a processing enzyme, Tatsumi *et al.* changed Ser228 to Ala by SDM [27]. *Saccharomyces cerevisiae* cells harboring a recombinant plasmid with mutant Alp did not secrete active Alp into the culture medium. The yeast cells accumulated a protein of 44 kDa, probably a precursor of Alp(the 34-kDa mature Alp plus the 10-kDa pro-peptide), suggesting that autoproteolytic processing of the pro-region was occurring. Introduction of a disulfide bond by SDM is known to enhance the thermostability of a cysteine-free enzyme. Aqualysin I, a thermostable subtilisin-type protease from *Thermus aquaticus* YT-1, contains four Cys residues forming two disulfide bonds [28]. The primary structure of Alp showed 44% homology to that of aqualysin I, and sites for Cys substitutions to form a disulfide bond were chosen in the Alp based on this homology. Ser69, Gly101, Gly169, and Val200 were replaced by Cys in the mutant Alp. Both Cys69-Cys101 and Cys169- Cys200 mutant Alps were expressed in *S. cerevisiae*, and the enzymes were purified to homogeneity. The Cys169-Cys200 disulfide bond was shown to increase the thermostability as well as the thermotolerance of *Aspergillus oryzae* Alp [29]. In vitro mutation of an aspartic acid residue predicted to be in the active site abolished the barrier activity of *S. cerevisiae* [30].

[III] DIRECTED EVOLUTION OF ENZYMES

This approach recently emerged as a key technology to generate enzymes with new and/ or improved properties that are extremely important for industrial applications [31]. This approach has provided a powerful tool for the development of enzymes with novel properties, even without requiring knowledge of enzyme structure and catalytic mechanisms. Jaeger *etal*, Amold *etal*, Petrounia *etal*, [31, 32, 33] have reviewed various aspects and examples of DE approaches for studying key properties of biocatalysts. The DE approach uses three approaches: DNA shuffling, random priming recombination and the staggered extension process (StEP). The DE starts with identification of a target enzyme followed by cloning of the corresponding gene. An efficient expression system is required before the target gene is subjected to random mutagenesis and/or in vitro recombination, thereby creating molecular diversity. This is followed by screening and identification of enzyme variants with the desired properties. However, use of the DE approach is still far from being widespread or adopted by many research laboratories using standard molecular biology techniques, as it is still unclear at present which strategy is the most efficient for the evolution of a desired property for a given protein [31]. However, some

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successful attempts have been made to evolve selective biocatalysts, such as lipase, aldolase, hydantoinase, esterase, aspartate aminotransferase, kanamycin nucleotidyl transferase, β-lactamase, β-galactosidase peroxidase, amidase and fucosidase, using the DE approach [31, 32]. Among the few reports on alkaline protease, subtilisin is the enzyme of choice for improving the catalytic behaviour of enzymes, using the DE approach. The total activity and organic solvent stability of subtilisin E from *B. subtilis* has been enhanced in aqueous dimethylformamide using the DE approach [34]. To demonstrate the utility of DE, Zhao *et al*,[35] used the StEP process to recombine a set of five thermostabilized subtilisin E variants identified during a single round of error-prone PCR mutagenesis and screening. Screening the StEP-recombined library yielded a subtilisin variant whose half-life was increased 50-fold at 50 °C, compared with wild-type subtilisin. In comparison, Ran et al, [36] reported the used of DE approach, using in vitro random mutagenesis and an improved screening method, to develop a cold-adapted mutant subtilisin from *B. subtilis* UOT0999 with a catalytic efficiency at 10 °C 100% higher than the wild-type strain. In another report, the thermal stability of subtilisin E was increased by converting it into a thermitase using DE [37]. This fascinating area of protein design will no doubt be the heart of future commitments in the enzyme industry.

3.1. A Flow Cytometry-Based Screening System for Directed Evolution of Proteases

Protein engineering, especially directed evolution, is a powerful tool and significant approach for protease reengineering. Directed protease evolution experiments produced proteases with improved catalytic efficiency such as cold adaption, increased thermostability, higher resistance toward oxidizing agents, activity in the absence of calcium ions, organic solvent resistance, and altered substrate specificity [38]. Some few reports employ protease inhibitors to probeactive sites of proteases to understand protease specificity [39, 40] or to access the potential of inhibitors as probable therapeutics [41].

Directed protease evolution campaigns conventionally use traditional screening formats such as agar plate (halo formation) or microplate-based detection systems[42]. The agar plate assays are usually applied in cases for a prescreening to identify the proteolytic activities at simple reaction conditions. The microplate-based screening methods are commonly used in directed evolution with capacities to be applied for more complex reaction cases. The two systems are usually associated with low mutational loads (one to three amino acid changes per mutated gene) so that a medium throughput is sufficient to find improved variants [43]. A notable example for a high-throughput screening (HTS) of protease variants is the directed evolution of the surface membrane protease OmpT to alter its substrate specificities by employing an *Escherichia coli* OmpT-deficient strain (UT5600)[44]. Improved variants were identified using flow cytometry in aqueous solution without employing any compartmentalization technology.

Miller *etal*, [45] reported recently, a (water-in-oil-in-water) double-emulsion-based compartmentalized flow cytometry screening technology (in vitro compartmentalization, IVC) has emerged as a potentially powerful screening format for sampling mutant libraries with approximately up to 108 variants per day. The IVC systems use a man made cell compartmentalization (e.g., w/o/w double-emulsion droplets), in which enzymatic reactions can be performed individually in femtoliter volume scale, resulting in a significant decrease of consumable cost. The principle of IVC-based flow cytometry screening systems employs fluorescence detection of a fluorescent product derived from a fluorogenic substrate. Therefore, the existence of a retainable fluorescence signal and its intensity are key parameters for the successful implementation of flow cytometry analysis employing IVC technologies [46]. In contrast to the surface adsorption method, the compartmentalized screening technology (IVC) is more generally applicable and could enable novel evolution strategies, for instance, by employing libraries with high mutational loads. Progress in IVC technology has been summarized well in several reviews [47, 48].

Ran *et al*, [38] created a first protease cytometry screening system (pro FC-IVC) which was developed by employing subtilisin Carlsberg as a model protease. Optimized parameters comprised (a) substrate selection to avoid partitioning of substrate and product into the oil phase, (b) host strain selection to minimize protease background reactions, and (c) host strain recovery after sorting. The developed protocol was subsequently validated by screening an epPCR library in which the mutational load was adjusted to 2%–3% of active clones.

In summary, Flow cytometry screening technology based on in vitro compartmentalization in double emulsion had been developed and applied on directed evolution of paraoxonase and β-galactosidase. In addition, advancements of flow cytometry– based screening technologies will enable an ultra-high throughput of variants offering novel opportunities in directed enzyme evolution under high mutational loads. For the industrially important enzyme class of proteases, a first flow cytometry–based screening system for directed protease evolution has been developed based on an extracellular protease-deficient *Bacillus subtilis* strain (WB800N), a model protease (subtilisin Carlsberg), and a water-in-oil-in-water double-emulsion technology. *B. subtilis* WB800N cells are incoporated in double emulsion with a fluorogenic substrate (rhodamine 110–containing peptide), paving way for the screening of protease variants in femtoliter compartments at high throughput. The protease screening technologyhas been validated by employing an epPCR mutant library with a high mutational load and screened for increased resistance toward the inhibitor antipain dihydrochloride. A variant (K127R, T237P, M239I, I269V, Y310F, I372V) with an improved

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relative resistance was isolated from a small population of active variants, validating the reported protease flow cytometry screening technology for increased inhibitor resistance [38].

[IV] ENZYMES ENGINEERING AND THEIR STABILITY IN SOLVENT

Reasearches all over the world for the past nearly three decades have unfolded the fact that, hostile environment provided to enzymes such as organic solvents, can catalyze reaction that were impossible in water, become more stable and exhibit new behavior such as "molecular memory". The proteins and/or enzymes stability is a major concern for their applications at industrial scales. High thermostable biocatalysts have a prolonged viability. For many biocatalysts, enzymatic reactions and stability of enzyme at high temperatures are pre-requisites for industrial use. However, the stability of enzyme in organic solvents, at extreme pH, pressure and stability towards mechanical disturbances are required for organic synthesis, chemical analysis, isolation and purification of chemicals, in therapeutics and diagnostics and in the study of protein structures and functions [36].

There has been persistent scientific effort to search for methods to prepare stable enzymes. Some of the examples of methods used for stabilizing microbial proteases are chemical modification using PEG, bio-imprinting of alpha (α) chymotrypsin in anhydrous media, chemical cross linking, molecular imprinting, immobilization in hydrophobic solvents, use of lyoprotectants [such as sugars, substrate-resembling ligands and crown ethers and protein engineering [36]. Inspite of the large number of research papers published on the organic-solvent stability of microbial proteases and peptide synthesis, only a few chosen methods have been adopted at industrial level. The primary reason being in ability of the rejected biocatalyst to have either of these two properties: substrate specificity, or sufficient organic-solvent stability. Thus, the discovery of novel microbial proteases having all the necessary required properties is considered extremely important for the use of enzymes on an industrial scale.

[V] CONCLUSIONS

Microbial proteases have numerous industrial and pharmaceutical applications. These applications are primarily achieved by engineering the enzymes using different approaches. The commercial successes of these enzymes lead to extensive study of biochemical, regulatory and molecular aspect of the enzymes system [26, 42]. The researchers have been and will continue to aim the discovery and engineering of novel microbial proteases that can perform efficiently at industrial scale. Currently, protein engineering of microbial proteases has been playing an important role in several industries. Different approaches such as SDM and DE will offer possibilities of generating proteases with entirely new function. The per suit for other newer approaches and/or strategies targeting new dimensions of molecular diversity and technology to improve performance characteristics by in vitro evolutionary changes of protein primary structures and high through put screening methods will continue to be the significant field of development in next few years.

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